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1 Multiplex PCR reveals unique trends in pathogen and parasitoid infestations of alfalfa leafcutting brood
2 cells.
3
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16 **Abstract**

17 The alfalfa leafcutting bee *Megachile rotundata* Fabricius (HYMENOPTERA: Megachilidae) is
18 an important pollinator for multiple agricultural seed commodities in the United States. *Megachile*
19 *rotundata* is a solitary bee that forms brood cocoons where its larvae can develop. During the
20 developmental stages of growth, broods can be preyed upon by multiple different fungal and bacterial
21 pathogens and insect predators and parasitoids, resulting in the loss of the developing larvae. Larval loss
22 is a major concern for alfalfa (*Medicago sativa* L.) seed producers because they rely on pollinator services
23 provided by *Megachile rotundata* and reduced pollination rates result in lower yields and increased
24 production costs. In the present study, we examined the taxonomic composition of organisms found
25 within *M. rotundata* brood cells using a multiplex PCR assay which was developed for the detection of
26 the most common bacterial, fungal, and invertebrate pests and pathogens of *M. rotundata* larvae. Known
27 pests of *M. rotundata* were detected, including members of the fungal genus *Ascosphaera*, the causative
28 agent of chalkbrood. Co-infection of single brood cells by multiple *Ascosphaera* species was confirmed,
29 with potential implications for chalkbrood disease management. The multiplex assay also identified DNA
30 from more than 2,400 total species including multiple new predators and pathogenetic species not
31 previously documented in associated with *M. rotundata* brood cells.

32

33 **Key words**

34 *Megachile rotundata*, multiplex PCR, *Ascosphaera*

35

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36 **Introduction**

37 The alfalfa leafcutting bee *Megachile rotundata* Fabricius (HYMENOPTERA: Megachilidae) is
38 an important pollinator for multiple agricultural commodities in the United States including alfalfa seed,
39 canola, melons and carrots. One of the primary agricultural commodities that uses *M. rotundata* for
40 pollination is alfalfa (*Medicago sativa* L.) seed production, which provides the germplasm for alfalfa used
41 for hay [1-5]. While alfalfa seed production is predominately limited to the Pacific Northwest region of
42 the United States, alfalfa hay production encompasses more than 17 million acres with a production value
43 of more than 8.8 billion dollars annually in the United States [5-6] and is only possible with the
44 production of high-quality seed. An insect pollinator is necessary to generate a seed crop in alfalfa, as
45 alfalfa is self-incompatible and unable to self-pollinate, and alfalfa seed producers rely on *M. rotundata*
46 and the ground dwelling alkali bee, *Nomia melanderi*, to fill this role [2,4,7-9]. The reliance on these bees
47 has resulted in *M. rotundata* being one of the most heavily managed pollinator species in the world [9].
48 *Megachile rotundata* is a solitary bee that constructs a nest (cocoon) for oviposition and larval
49 development. A single female *M. rotundata* will construct a nest that consists of individual brood cells
50 that are lined with cut leaves (cocoon) [9,10-11] and filled with pollen to provide nutrition for the
51 developing larva. After construction, a female leafcutting bee will lay a single egg within the cell and
52 seal the egg within the cocoon to undergo larval stage development and pupation into the adult stage [9].
53 This cocoon construction requires multiple trips to flowering plants and makes alfalfa leafcutting bees a
54 highly effective pollinator species [1,9]. As with most pollinating species, including honey bees (*Apis*
55 *sp.*), mason bees (*Osmia bicornis*), and blue orchard bees (*Osmia lignaria*), *N. melanderi* and *M.*
56 *rotundata* are preyed upon by predatory insects and play host to numerous fungal pathogens [12-16]. The
57 presence of invertebrate pests and fungal pathogens within *M. rotundata* brood cells is a major concern
58 for alfalfa seed producers, as they can cause reduced pollination efficiency by resulting in brood loss.
59 Multiple pathogens and parasitoids can infect a developing larva, often resulting in the loss of brood.
60 Common fungal pathogens include multiple *Ascosphaera* species, which cause chalkbrood. Alfalfa seed
61 growers are predominately concerned with the presence of *Ascosphaera aggregata* within brood cells, as
62 it is currently thought to be the major *Ascosphaera* species which results in *M. rotundata* brood loss [17].
63 However, multiple different *Ascosphaera* species have been detected in *M. rotundata* nest cells. For
64 example, *A. subglobosa*, *A. acerosa*, *A. asterophora*, *A. larvis, A. pollenicola,* and *A. proliperda* [18-21]
65 have all been found in *M. rotundata* brood cells and can cause the chalkbrood phenotype. Broods may
66 also be attacked by other insects, including parasitic wasp species (such as *Monodontomerus obscurus*,
67 *Leucospis affinis*, *Pteromalus venustus*, and *Sapyga pumila* [22]), nest destroying beetles (including
68 *Tribolium audax*, *Tribolium brevicornis*, and *Trichodes ornatus* [22]), and cuckoo bees (*Epeoloides*
69 *pilosula*) [22], all of which feed on developing larvae and further decimate producers' bee stocks. The

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70 presence of these pathogens and parasitoids can significantly impact the pollination of the alfalfa seed
71 crop and subsequently decrease the crop yield by reducing pollinator populations and efficiency.
72 Additionally, these pathogens and predators can reproduce within grower stocks, bee boards, and housing,
73 and if not controlled, can result in a high abundance of dead bee larvae. With these bees being heavily
74 managed, sold, and transported throughout growing regions, this can cause significant concern for
75 growers.

76 In order to reduce brood loss, growers use a combination of disinfectants and lures to protect
77 brood from different pathogens and parasitoids [23-26]. Growers monitor the presence of these pathogens
78 using X-ray (Faxitron) imaging as a diagnostic technique [27]. With current detection practices limited to
79 X-ray diagnostics and few published polymerase chain reaction (PCR) amplifications of *Ascosphaera*
80 species [28], we set out to examine the taxonomic makeup of *M. rotundata* brood cells through a
81 multiplex deep sequencing PCR reaction. The multiplex was designed to amplify short sequence reads
82 (150-200bp) to known and unknown pathogen, predators, and *Ascosphaera* species. If primers are
83 designed correctly, multiplex technology can provide millions of short sequences which can be mapped
84 back to reference sequences revealing the identity and composition of all species within a sample. As
85 such, this technique can provide a glimpse into the complex biological niche of the *M. rotundata* brood
86 cell.

87 In the current investigation we examined the taxonomic makeup of *M. rotundata* brood cells to
88 determine if we could generate a multiplex PCR reaction that could confirm the presence of known
89 pathogens and potentially identify new pathogens present in *M. rotundata* brood cells. Currently,
90 diagnostic techniques rely on the visual assessment of pathogen and predator infection using Faxitron (X-
91 ray) imaging. Pathogens and predators can be challenging to identify and classify using imaging alone. In
92 order to develop a new, more definitive, and more sensitive method to detect infection, we explored the
93 use of an Illumina-based DNA multiplex PCR assay. To develop this assay, we classified the presence of
94 different pathogens and predators within 4 populations of *M. rotundata* cells encompassing 4 grower
95 populations from Idaho and Washington using Faxitron imaging. We extracted total DNA from a set of
96 200 *M. rotundata* brood cells encompassing healthy brood cells, pathogen/predator infested brood cells,
97 and brood cells with unknown infection status. Using an Illumina-based multiplex PCR assay we
98 examined taxonomic makeup of the brood cells, including known and novel pathogens and predators. The
99 analysis resulted in the identification of multiple, new potential pathogens of *M. rotundata* bee cells.
100 Further, the multiplex analysis provided insight into which species of *Ascosphaera* can infect *M.*
101 *rotundata* brood cells, including the detection of multiple *Ascosphaera* species co-infections within
102 individual brood cells.
103

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104 ## Materials and methods

105

106 ### Data availability

107 All relevant data are contained within the paper and its supporting information files.

108

109 ### Ethical statement

110 This article does not contain studies with any human participants and no specific permits were required
111 for collection or experimental treatment of *Megachile rotundata* for the study described.

112

113 ### *Megachile rotundata* sample collection

114 *Megachile rotundata* samples were acquired from growers as part of the Parma Cocoon
115 Diagnostic Laboratory service (Parma, Idaho). This service examines bee health for alfalfa seed growers
116 through Faxitron diagnostics. The Parma Cocoon Diagnostic Laboratory (PCDL) is an extension-oriented
117 service that classifies the proportion of pathogen and parasitoid infected *M. rotundata* brood cells
118 submitted by growers. PCDL uses X-ray imaging to visually classify brood cell fungal pathogens
119 including *Ascosphaera aggregata* and *Ascosphaera larvis*, insect parasites including imported chalcid
120 wasps (*Monodontomerus obscurus*), cuckoo bees (*Epeoloides pilosula*), woodboring chalcid wasps
121 (*Leucospis affinis*), long-tongued blister beetles (also known as sunflower beetles, *Nemognatha lutea*),
122 Canadian chalcid wasps (*Pteromalus venustus*), and red-marked sapygids (*Sapyga pumila*), and
123 predators/nest destroyers including American black flour beetles (*Tribolium audax*), giant flour beetles
124 (*Tribolium brevicornis*), and checkered flower beetles (*Trichodes ornatus*). Brood cells are shipped
125 directly to the PCDL from commercial growers as a loose aggregate. Samples used within this experiment
126 were acquired from multiple growers from the Pacific Northwest. Grower information cannot be
127 disclosed as part of a confidentiality agreement. Upon arrival, samples were transferred to a sterile
128 incubator held at 27˚C. Samples were used in both Faxitron and multiplex DNA analysis.

129

130 ### Faxitron analysis

131 Using sterile laboratory techniques, individual brood cells from each grower sample were lined
132 up on a piece of contact film. A total of five, 10-gram samples for each population were X-rayed using a
133 Faxitron (Model 42855A) machine. Samples were X-rayed using Kodak Industrex-M radiographic film.
134 Samples were processed at 20kVp for a 1-minute exposure time. Samples were visually analyzed using
135 defined standards for fungal pathogens, invertebrate pests, dead larvae, pollen balls, and healthy larvae.
136 After the brood cells were classified, they were removed from the sticky paper using sterilized tweezers
137 and placed in sterile 1.5ml microcentrifuge tubes (visual classification of cells can be found in

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138 **Supplemental File S1**). To minimize any contamination within samples, all laboratory equipment was
139 sterilized before and after contact with brood cells with 95% ethanol and a Bunsen burner.
140

141 ### *Megachile rotundata* brood cell DNA extraction

142 DNA was extracted from individual cells using a modified CTAB extraction protocol. Individual
143 brood cells were placed in 2ml DNAse/RNAse-free homogenate tubes (Biospec, OK, USA) with a single
144 sterilized 6.4mm diameter glass bead (Biospec, OK, USA). Seven hundred and fifty µl of CTAB
145 extraction buffer was added to each tube (OPS diagnostics, NJ, USA). Samples were homogenized for 2
146 minutes in a Mini-beadbeater-16 (Biospec, OK, USA). Tubes with homogenate were incubated at 60°C in
147 a water bath for 30 minutes. Following the incubation period, samples were centrifuged for 10 minutes at
148 14,000 x g and the supernatant was transferred to new 1.5ml tubes. Five µl of RNase solution A
149 (20mg/ml, Fisher Scientific, MA, USA) was added and incubated at 37°C for 20 minutes. Three hundred
150 µl of chloroform/isoamyl alcohol (24:1) was then added to each sample and vortexed for 5 seconds, then
151 centrifuged for 1 minute at 14,000g to separate the phases. The chloroform/isoamyl alcohol step was
152 conducted twice. The upper aqueous phase was transferred to a new 1.5ml microcentrifuge tube. DNA
153 was precipitated by adding 500µl cold isopropanol. Samples were left for 12 hours at -20˚C. Samples
154 were centrifuged at 14,000g for 10 minutes to pelletize DNA. Supernatant was decanted without
155 disturbing the pellet and was subsequently washed with 1ml of ice-cold 70% ethanol, and the samples
156 were vortexed and centrifuged at 14,000g for 10 minutes. Ethanol was decanted and excess ethanol was
157 removed from the pellet with a pipettor. Samples were air dried in a sterile PCR cabinet for 15 min. DNA
158 was dissolved in 100µl RNase/DNase-free H_2O. DNA concentrations were determined using a Nanodrop
159 2000 (Thermo Fisher Scientific, MA, USA). Samples were stored at -20 C until multiplex processing.
160

161 ### *Megachile rotundata* brood cell multiplex PCR and data analysis

162 Total DNA from each sample was sent to Floodlight Genomics LLC (TN, USA). Floodlight
163 Genomics used an optimized Hi-Plex approach to amplify targets in a single multiplex PCR reaction [29].
164 Primers were designed to amplify both known fungal pathogens (*A. aggregata* and *A. larvis,* as they are
165 the two species of *Ascosphaera* screened by the Parma Diagnostic Laboratory), known invertebrate pests,
166 and unknown bacteria (**Supplemental File S2**). The primers were also intentionally designed over large
167 regions of genes used in phylogenetic investigations including cytochrome C oxidase, 18S ribosomal
168 RNA, 28S ribosomal RNA and bacterial V regions to be able to examine the overall taxonomic makeup
169 of the *M. rotundata* brood cell. The sample-specific barcoded amplicons were sequenced on the Illumina
170 HiSeq X platform according to the manufacturer's directions. Floodlight Genomics delivered sample-
171 specific short raw DNA sequence reads as FASTQ files. Annotation of the raw reads was performed with

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172 Geneious Bioinformatics Software (Auckland, New Zealand). Reads were grouped by both individual
173 brood cell and as an aggregate of all brood cells. Raw reads were mapped to reference sequences at 100%
174 stringency for classification. Raw reads were also assembled (as an aggregate of all samples) into
175 biological contigs using Geneious (**Supplemental File S3**). Assembled contigs were uploaded into
176 Blast2go (BioBam Bioinformatics, MA, USA) for analysis. The National Center for Biotechnology
177 Information (NCBI) nucleotide database was downloaded in May of 2021. The database was uploaded
178 into Blast2go to generate a refence database. Contigs were blasted against the reference database with a E
179 value cut of 10^{-10}. Taxonomic identification of contigs was determined using R version 4.0.4 and the
180 *taxonomizr* package [30]. Phylogenic trees were generated based on NCBI scientific name through
181 phyloT v2 database 2021.1 [31].
182
183 ***Ascosphaera* DNA extraction**
184 An additional set of *M. rotundata* brood cells were classified as being infected with *Ascosphaera*
185 (via X-ray diagnostics) and were placed within sterile 1.5ml centrifuge tubes. Brood cells were sterilized
186 prior to DNA extraction by washing with a sterilization solution designed to sterilize and remove
187 contaminants from the outside of the *M. rotundata* brood cells. The sterilization solution comprised 5%
188 190 proof Ethanol, 1% Tween 20 (ThermoFisher Scientific, Waltham, MA, USA), and 0.1% D-256
189 (Venco, St. Joseph, MO) and was generated in DNase/RNase-free water (ThermoFisher Scientific,
190 Waltham, MA, USA). Aliquots of 1ml of sterilization solution were placed into 1.5 ml microcentrifuge
191 tubes, and new aliquots were used for every brood cell. Brood cells were dipped in the sterilization
192 solution aliquot using forceps for 30 seconds moving up and down within the solution. Cells were then
193 washed with DNase/RNase-free water to remove any residues. Sterilized brood cells were then placed in
194 sterile 2ml DNAse/RNAse-free homogenate tubes (Biospec, OK, USA) and allowed to completely dry
195 before DNA extraction in a sterile PCR cabinet. DNA was extracted from individual cells using a DNeasy
196 Plant Pro Kit (Qiagen, Hilden, Germany). The DNeasy Plant Pro Kit requires limited steps compared to
197 the CTAB method, resulting in less chance to contaminate the purified DNA. DNA concentration was
198 determined using a Nanodrop 2000. Samples were stored at -20°C until PCR and multiplex processing.
199
200 ***Ascosphaera* traditional PCR**
201 To confirm the presence of *Ascophaera* from DNA isolations, a PCR amplification was
202 conducted on a subset of the DNA extractions using primers designed by James and Skinner 2005 [28].
203 Three PCR reactions were conducted to amplify all *Ascosphaera* species, group 1 (*A. aggregata* and *A.*
204 *proliperda*), and group 2 (*A. columbrina*, *A. variegata*, *A. larvis*, and *A. pollenicola*) (Primers can be
205 found in **Supplemental file S2**). Specifically, 25µl PCR was conducted with GoTaq Green Master Mix

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206 (Promega Corporation, Madison, WI). Reaction conditions included 240s at 94°C as the initial denaturing
207 step, followed by 40 cycles of 60s at 94°C for denaturation, 60s at 63°C for annealing, 60s at 72°C for
208 extension and a final extension of 300s at 72°C. PCR amplification products were separated on a 1.5%
209 agarose gel to confirm presence of corresponding DNA fragments.
210

211 ***Ascosphaera* multiplex PCR**

212 Twenty IST1-5.8S-IST2 sequences from Anderson et al. 1998 [32] were downloaded from NCBI,
213 the IST2 region of the sequences was aligned using Geneiuos Prime tree builder global alignment (**Figure**
214 **1**). From the alignment we determined that the IST2 region had significant sequence differences that
215 could be used to classify *ascosphaera* species. Primers were designed to amplify the IST2 region of
216 *Ascosphaera* (**Supplemental File S2**). Total DNA from 38 *Ascosphaera* samples were sent to Floodlight
217 Genomics LLC. Floodlight Genomics used an optimized Hi-Plex approach to amplify targets in a single
218 multiplex PCR reaction [29]. The sample-specific barcoded amplicons were sequenced on the Illumina
219 HiSeq X platform according to the manufacturer's directions. Floodlight Genomics delivered sample-
220 specific short raw DNA sequence reads as FASTQ files. Annotation of the raw reads was performed with
221 Geneious Bioinformatics Software (Auckland, New Zealand). Reads were treated as individual cells and
222 mapped to reference sequences with a minimum overlap identity of 95% and reads only mapping to the
223 single best match of the 20 reference sequences was allowed. A sample was considered infected with an
224 *Ascosphaera* species if there were more than 1000 sequence hits. The high threshold of 1000 was used to
225 eliminate the chance that a cell was not completely sterilized before extraction.
226

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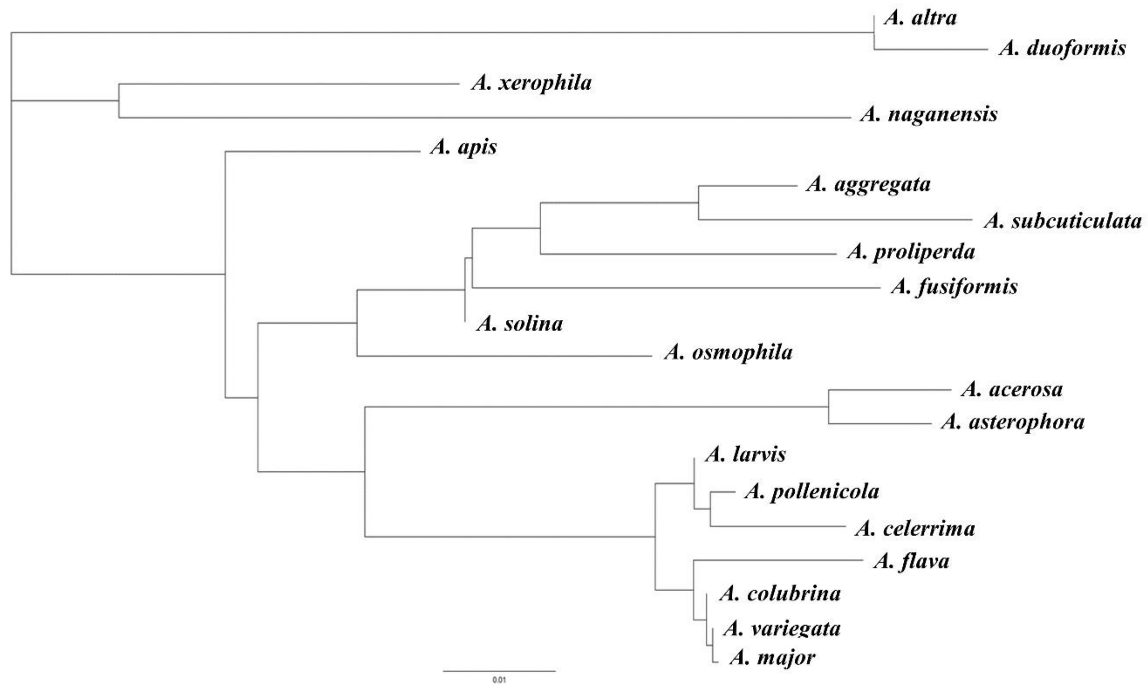
227



228

229 **Figure 1.** IST2 sequences alignment from *ascosphaera* species obtained from Anderson et al. 1998 [32]

230

231 ## Results

232

233 ### *Megachile rotundata* brood cell multiplex PCR

234 A total of 21,974,525 short Illumina reads were acquired from Floodlight Genomics and mapped
235 to a set of reference sequences to known pathogens and parasitoids of *M. rotundata* cells. In total 607,769
236 reads were successfully mapped to a reference sequence (100% overlapping identity). Only *A. aggregata,*
237 *A. larvis, T. ornatus,* and *T. brevicornis* were successfully identified within the samples using this
238 method. *A. larvis* and *A. aggregata* were found within samples from each population analyzed. *T. ornatus*
239 was classified in population 1 and *T. brevicornis* was classified in population 2. We noted that almost all
240 samples had DNA which corresponded to both *A. aggregata* and *A.larvis*. This was most likely due to
241 how samples were processed and shipped as aggregates, resulting in both fungal and invertebrate DNA
242 contamination throughout the entire sample. While within regional samples it is possible to determine
243 taxonomic classification, it was not possible within individual cells for this reason.

244

245 ### BLAST assembly

246 To further explore the taxonomic makeup of the *M. rotundata* brood cells, we aggregated all
247 reads into a single sample. A total of 51,056 contigs were assembled using Geneious Prime. The assembly

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248 was conducted using standard cutoffs and conditions provide by the software. The contigs were compared
249 against the entire NCBI nucleotide database to investigate taxonomic composition of *M. rotundata* cells
250 and explore new predators and pathogens of *M. rotundata* cells. This composition was conducted as an
251 aggregate of all brood cells to examine the general makeup of these complex biological systems. In total
252 there were 2,438 different species classified in the analysis (**Supplemental File S4**), including 13
253 archaeal, 1,716 bacterial, and 709 eukaryotic contigs which could be differentiated into biological classes
254 (**Supplemental Figure S5**). The predominant orders of insects were Hymenoptera and Coleoptera
255 (**Figure 2**). Contigs within the class Insecta belonged to multiple different parasitic wasps, bees, and nest
256 destroying beetles. These include various parasitic wasp taxa (Chalcididae, Eupelminae, *Melittobia spp.*,
257 *Mesopolobus spp.*, *Pteromalus spp.*, *Torymus spp.*, and *Pediobius spp.*), the cuckoo bee (*Coelioxys*
258 *pieliana*), and predator/nest destroyers *Tribolium audax* and *Tribolium brevicornis*. Additional parasitic
259 wasp taxa that infest aphids and other non-*M. rotundata* insects were also observed, including
260 *Diaeretiella spp., Encarsia spp.*, *Lysiphlebus spp.*, and *Pauesia spp.*

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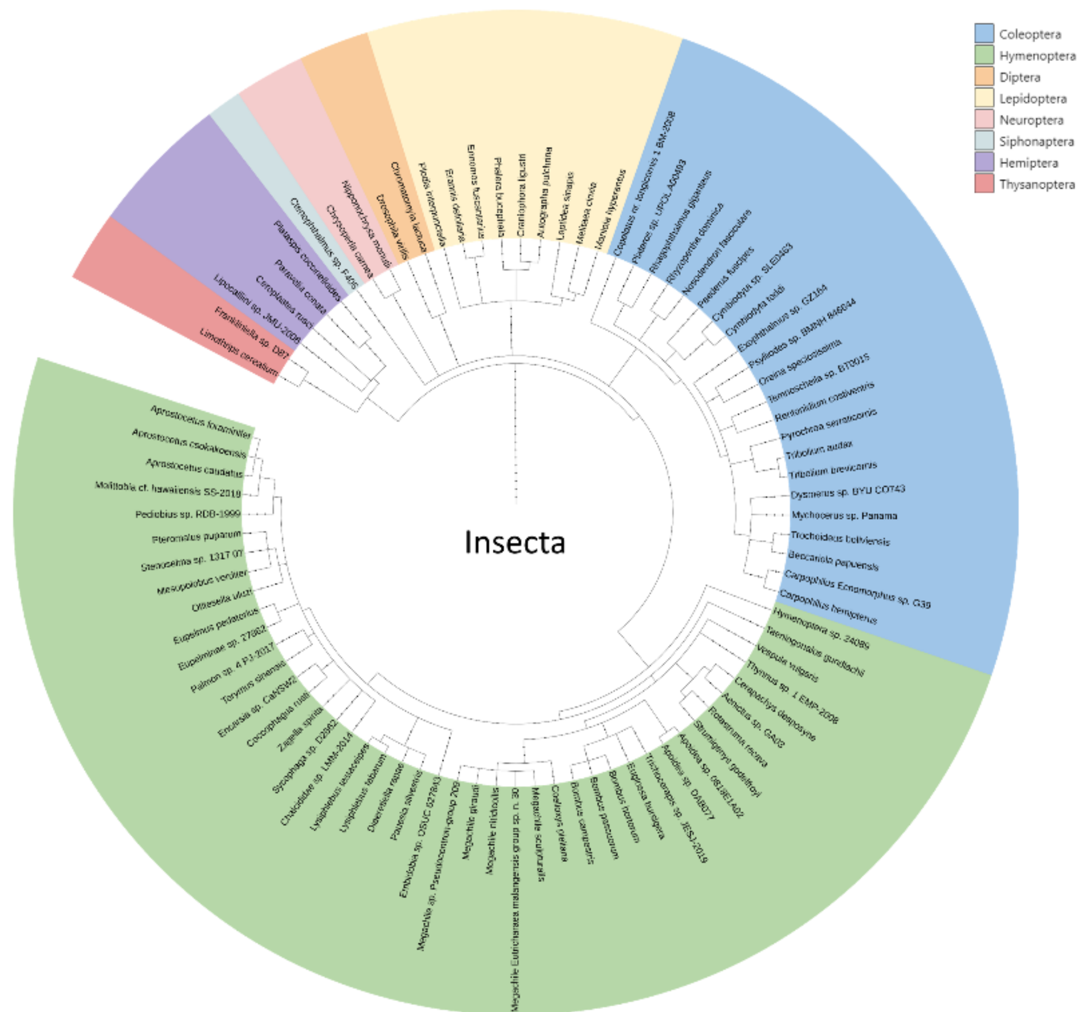

261

262

263 **Figure 2.** Insect species and orders of contigs found within *M. rotundata* brood cells

264

265 We further investigated the division of fungal ascomycetes found within *M. rotundata* brood
266 cells. Members of the fungal division Ascomycota (**Figure 3**) are known as sac fungi and include
267 multiple known pathogens of bees within the class Eurotiomycetes. The contig assembly revealed
268 multiple different known human and insect pathogens including *Aspergillus* and 20 *Ascosphaera* species.
269 *Aspergillus* species, which are known mammalian pathogens, are most likely a product of decomposing
270 plant tissue within the brood cocoon. *Ascosphaera* species are known pathogens of insects and could be
271 predating on and reproducing within the brood cells.

272

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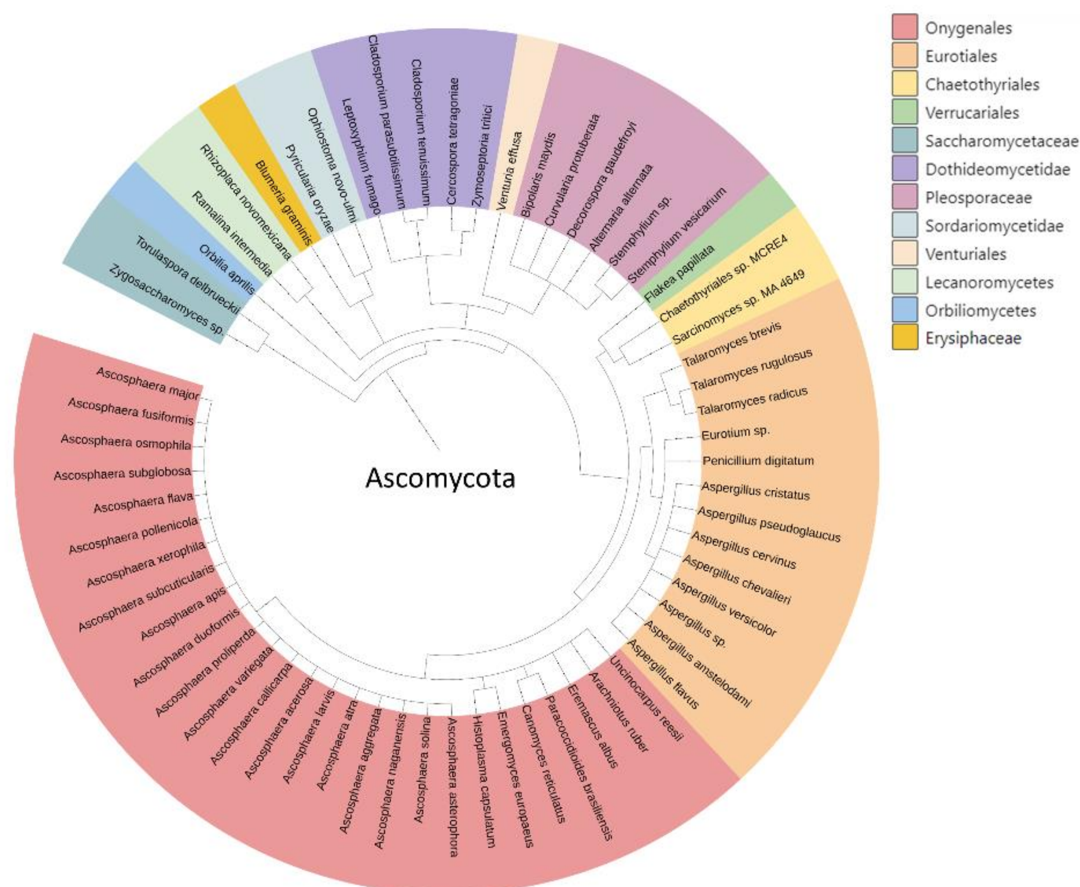

273 *Unable map the Curvularia umbiliciformis, Neopetractis nodispora, Huriella flakusii, and Lilapila oculisporella

274 **Figure 3.** Fungi division Ascomycota designation of contigs found within *M. rotundata* brood cells

275

276 ***Ascosphaera* data analysis**

277 PCR revealed the presence of co-infected of *M. rotundata* cells by multiple different *Ascosphaera*
278 species. A subset of 18 *Ascosphaera* DNA extractions were PCR amplified to confirm the presence of
279 *Ascosphaera* within the *M. rotundata* sample. For each *Ascosphaera* DNA extraction, three PCR
280 amplifications were conducted. The first PCR amplification used non-specific primers which amplify
281 most *Ascosphaera* species [28], which revealed that 13 out of the 18 samples were positive for
282 *Ascosphaera* (**Figure 4a**). The second amplification which was specific for the amplification of *A.*
283 *columbrina*, *A. variegate*, *A. larvis*, and *A. pollenicola* revealed that that 12 of the 18 samples were
284 positive for one or more of these species (**Figure 4b**) and the final PCR amplification which was specific
285 to *A. aggregata* and *A. proliperda* revealed 13 out of the 18 samples were positive for one or both of these
286 species (**Figure 4a**). The PCR amplification also indicated that most samples (12 in total) had the
287 presence of multiple different *Ascosphaera* species. The size of the bands from the second amplification
288 also indicate that the primers may not only be specific to *Ascosphaera* for this amplification. A negative

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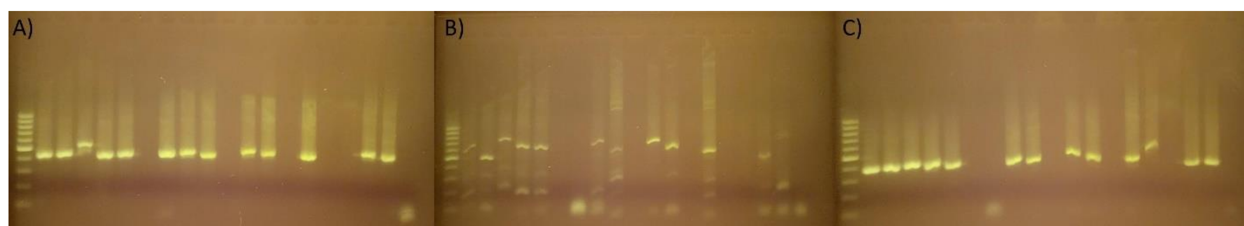
289 control was run with all PCR amplification and is depicted as the last well in each image, which had no
290 amplification.
291



292
293 **Figure 4.** Amplification of *Ascosphaera* from *M. rotundata* cells on a 1.5% agarose gel with a 1kb DNA
294 marker A) general *Ascosphaera* amplification, B) *A. columbrina*, *A. variegate*, *A. larvis*, and *A.*
295 *pollenicola* specific PCR and C) *A. aggregata* and *A. proliperda*.
296
297 Deep sequencing of the IST2 region of *Ascosphaera* revealed the identification of the species of
298 *Ascosphaera* infecting *M. rotundata*. The amplification of the IST2 region resulted in the amplification of
299 *A. aggregata, A. atra, A. duoformis, A. naganensis, A. proliperda,* and *A. solina.* Only one sample was not
300 co-infected with multiple different *ascosphaera* species which was only infected with *A. aggregata*.
301 Further, *A. Larvis, A. acerosa, A. asterophora* was detected within the IST2 region amplification however
302 were below the classified threshold for detection.
303
304 **Table 1.** Multiplex amplification of *M. rotundata* cells infested with *ascosphaera*

	A. aggregata		*A. atra*		*A. duoformis*		*A. naganensis*		*A. proliperda*		*A. solina*	
Sample	Reads	% of Reads	Reads	% of Reads	Reads	% of Reads	Reads	% of Reads	Reads	% of Reads	Reads	% of Reads
Cell 94	5820	100	0	0	0	0	0	0	0	0	0	0
Cell 104	21782	85.44	0	0	0	0	3712	14.51	0	0	0	0
Cell 129	16991	32.08	0	0	0	0	3081	5.81	25134	47.41	7766	14.64
Cell 130	36120	81.74	0	0	0	0	5980	13.51	0	0	2090	4.72
Cell 132	5564	25.69	0	0	0	0	1003	4.63	11613	53.55	3476	15.99
Cell 136	15975	84.71	0	0	0	0	2884	15.22	0	0	0	0
Cell 138	24325	85.32	0	0	0	0	4186	14.64	0	0	0	0
Cell 197	0	0	46969	95.29	2321	4.70	0	0	0	0	0	0
Cell 266	0	0	9321	55.75	5360	31.95	0	0	2038	12.13	0	0
Cell 323	0	0	25708	8.47	2768	0.91	1311	0.43	266622	87.84	7123	2.35
Cell 331	0	0	0	0	0	0	0	0	46261	96.36	1747	3.63
Cell 360	0	0	0	0	0	0	1357	0.57	228693	96.30	7425	3.13
Cell 364	11558	31.38	0	0	0	0	2005	5.44	19986	54.21	3284	8.89
Cell 679	4696	67.37	0	0	0	0	0	0	1179	16.75	1095	15.52
Cell 682	15333	55.77	1637	5.94	0	0	2381	8.64	5757	20.89	2385	8.65
Cell 683	11330	51.78	5030	22.93	0	0	2012	9.16	1591	7.24	1918	8.73

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Cell 684	11778	22.80	33897	65.58	4051	7.83	1936	3.74	0	0	0	0
Cell 685	2583	23.89	8227	75.94	0	0	0	0	0	0	0	0
Cell 689	13910	65.87	2651	12.52	0	0	1789	8.44	0	0	2766	13.05
Cell 691	25997	74.26	3500	9.98	1329	3.79	4182	11.92	0	0	0	0
Cell 692	6645	76.84	0	0	0	0	0	0	0	0	2003	22.96
Cell 693	13991	60.54	3758	16.22	1168	5.04	1965	8.47	0	0	2228	9.60
Cell 698	18027	76.13	0	0	0	0	2465	10.38	0	0	3186	13.41
Cell 724	17504	80.54	0	0	0	0	2513	11.52	0	0	1716	7.86
Cell 733	7239	77.29	0	0	0	0	0	0	0	0	2127	22.52
Cell 734	17893	66.09	0	0	0	0	2590	9.54	1389	5.12	5200	19.15
Cell 754	8993	26.07	0	0	0	0	1075	3.11	18426	53.36	6008	17.37
Total Reads per species	308234		140698		16997		48427		628689		63543	

Discussion

The continued health and protection of *M. rotundata* is vital for the success of the alfalfa seed, alfalfa hay, and livestock industries. While *M. rotundata* larvae develop within their cocoon produced by the mother bee they can still be preyed upon by multiple different parasitoids, pathogens, and nest destroyers. Classification of the different pathogens, parasitoids, and predators has traditionally revolved around visual classifications and X-ray diagnostics. Within this investigation we set out to examine the taxonomic makeup of *M. rotundata* brood cells and developed a multiplex PCR assay to examine the different *Ascosphaera* species that can infect *M. rotundata* brood cells and classified multiple potential new pathogens using this technique. We also found that developing brood can be co-infected my multiple *Ascosphaera* species.

We examined cells for the presence of known parasites, predators, nest destroyers, and fungal pathogens by first compiling genomic sequences from NCBI based on Evan et al. 1980 [22], which provided current known parasitoids and pathogens of *M. rotundata* brood cells. From the sequences we designed PCR primers and conducted a multiplex PCR reaction to confirm and classify known pathogens, parasites, predators, and nest destroyers of *M. rotundata* [19] within our samples to better understand the taxonomic composition of *M. rotundata* brood cells. Approximately 600,000 reads were successfully mapped to compiled references sequences, and these reads revealed the presence of *A. aggregata, A. larvis, T. ornatus, and T. brevicornis* within our *M. rotundata* brood cell samples. After this mapping, there were over 21 million reads that remained unmapped. To explore unmapped reads and examine the taxonomic makeup of the *M. rotundata* cell, we chose to assemble the short reads into contigs and BLAST against the entire NCBI database.

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329 The BLAST result revealed the diversity of the composition of *M. rotundata* cells, resulting in
330 2,438 different species classified. It is important to note that this investigation could not differentiate
331 between environmental contaminants within the samples. Environmental DNA could have been picked up
332 from the foraging mother *M. rotundata* from plant material as she provisioned her nest cells, including
333 fecal material deposited by other insects. Understanding the limitations of this investigation, we chose to
334 examine likely new fungal pathogens and insect predators and parasites. Within our BLAST investigation
335 we noted the presence of more than 20 *ascosphaera* species. We further examined the presence of
336 invertebrate pests and noted multiple species that have the potential to predate on insects, including bees.
337 These included parasitic wasps, the cuckoo bee (*Coelioxys pieliana*) and predator/nest destroyers
338 *Tribolium audax* and *Tribolium brevicornis*. However, with multiple species lacking sequence data in
339 NCBI, it is hard to make a definitive identification, and an approach of this manner might be more useful
340 to provide class and family identifications and generate direction for future investigations, including
341 providing insight into future sequencing endeavors to produce more comprehensive data for taxonomic
342 classification.
343 One interesting result from the BLAST analysis was abundance of different *Ascosphaera* species
344 found associated with *M. rotundata* cells. To better classify the identity of the *Ascosphaera* species
345 causing chalkbrood in *M. rotundata,* we decided to sequence the ITS2 region of the IST1-5.8S-IST2
346 nucleic acid sequence. Our findings suggest that *M. rotundata* brood cells can be infected by multiple
347 different *Ascosphaera* species, including being co-infected by multiple *Ascosphaera* species at the same
348 time. From this analysis we noted that most cells examined were co-infected with multiple different
349 *Ascosphaera* species including, *A. aggregata*, *A. atra*, *A. duoformis*, *A. naganensis*, *A. proliperda*, and *A.*
350 *solina*. We also noted the presence of *A. larvis, A. acerosa, A. asterophora* within the samples, however
351 they were below the detection threshold cutoff. Most samples were infected with *A. aggregata*, which is
352 the major concern for alfalfa seed growers. However, 5 of the samples had no *A. aggregata* detected
353 within the samples (above the detection threshold) and were primarily infected with *A. atra* or *A.*
354 *proliperda*. The findings suggest that other species of *Ascosphaera* besides *A. aggregata* could be
355 contributing to chalkbrood within *M. rotundata*. Our study was limited to a subset of infected *M.*
356 *rotundata* brood cells, and there is a possibility that other brood cells, particularly from different growing
357 regions, could be infected with different *Ascosphaera* species which were not detected within this
358 investigation. Further, the current genomic resources are limited regarding sequence data of *Ascosphaera*
359 species, and we based our assessments of our IST2 multiplex reaction only on reference sequences from
360 Anderson et al. 1998 [32] who sequenced different *Ascosphaera* species from pure cultures growing on
361 slanted solid agar media. It is possible that our alignments may be matching to unclassified *Ascosphaera*
362 species. This suggests the need for more in-depth genomic data for this agriculturally relevant species.

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363 From our data, it is clear that multiple *Ascosphaera* species can infect *M. rotundata* cells, including
364 simultaneous co-infection. More research should be conducted to determine how the interactions between
365 different *Ascosphaera* species may be affecting their pathogenicity.

366 This investigation revealed the complex taxonomic diversity of *M. rotundata* brood cells, with
367 more than 2,400 different known species found inside the brood cell including species of plants, fungi,
368 bacteria, archaea and insects. We further identified multiple different species that have the potential to
369 predate on *M. rotundata* that should be examined in more detail, including species of *Ascosphaera* that
370 could cause the disease phenotype known as chalkbrood. Pollinators will continue to be a vital resource
371 for alfalfa seed producers so the agricultural community and growers should be on the lookout for new
372 and emerging pathogens and parasitoids of leafcutting bees. We hope this investigation has provided
373 useful new methods and data to support that goal.

374

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378

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452 **Supporting information**

453 **Supplemental File S1:** *Megachile rotundata* bee cells classification used in multiplex analysis

454 **Supplemental File S2:** *Megachile rotundata* cells Multiplex, Ascosphaera PCR, and Ascosphaera cells
455 Multiplex primers

456 **Supplemental File S3:** *Megachile rotundata* bee contigs assembled by Geneious primer

457 **Supplemental File S4:** Taxonomic classification of contigs

458 **Supplemental File S5:** Taxonomic tree of class identification of *Megachile rotundata* bee cells